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Application of: Geoffrey Goldspink	Confirmation No.: 8321
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DECLARATION OF GEOFFREY GOLDSPINK UNDER 37 C.F.R. § 1.131

Mail Stop Amendment
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Sir:

I, GEOFFREY GOLDSPINK, declare the following:

1.   I reside at Brambledene, East Common, Harpenden AL5 1DQ, United Kingdom.

2.   I presently hold the positions of Emeritus Professor of Surgery and Emeritus Professor of Anatomy at University College London. University College London is the assignee of the above-identified application by virtue of an assignment from the inventor to University College London for International Application No. PCT/GB98/01198, to which the above-identified application claims benefit.

3.   I am the sole inventor of the invention which is disclosed and claimed in the above-identified application. The invention as described and claimed in the above-identified application relates to methods of treatment using pharmaceutical compositions comprising a) an expression cassette operably linked to (i) a myosin light chain enhancer; (ii) a promoter selected from a myosin heavy chain promoter and a viral promoter; and (iii) a polynucleotide sequence encoding a polypeptide of therapeutic use, b) a vector comprising said expression cassette; or c) a viral strain comprising said expression cassette combined with a pharmaceutically acceptable carrier or diluent.

4.   Attached hereto as Exhibit 1 is a copy of a draft manuscript that served as an Invention Disclosure Form for the invention described and claimed in the above-identified application. I have reviewed Exhibit 1. Although the dates have been removed from this document, the date of this document is prior to March 1997. Also, I hereby confirm that Exhibit 1 describes work done by me or under my direction and supervision and all of the acts relied upon in Exhibit 1 and this Declaration were carried out by myself or under my direction in the United Kingdom prior to March 1997.

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5.   As shown in Exhibit 1, prior to March 1997, I developed a plasmid expression vector comprising (i) a myosin light chain enhancer (e.g., the myosin light chain 1/3 enhancer); (ii) a promoter selected from a myosin heavy chain promoter (e.g., a truncated rabbit β-cardiac myosin heavy chain enhancer) and a viral promoter (e.g., CMV promoter); and (iii) a polynucleotide sequence encoding a polypeptide of therapeutic use (e.g., the α-galactosidase polypeptide) (see page 7 for description of constructs). Myoblast cells were transfected with the expression vector and successfully expressed and secreted α-galactosidase.

6.   Therefore, Exhibit 1 demonstrates that, prior to March 1997, I had conceived and I or persons acting at my direction had reduced to practice the claimed invention.

7.   I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Date   23rd March 2005

GEOFFREY GOLDSPINK

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DEVELOPMENT OF PLASMID VECTORS FOR THE PRODUCTION OF HUMAN ALPHA-GALACTOSIDASE FROM MUSCLE *IN VIVO* AND *IN VITRO*

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RUNNING TITLE: <u>VECTORS FOR MUSCLE EXPRESSION OF ALPHA-GALACTOSIDASE</u>.

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ABSTRACT

Deficiency of alpha-galactosidase results in Fabry disease, a lysosomal storage disorder. We have investigated the expression and secretion of human alpha-gal from mouse C2C12 myoblasts after liposome-based transfection with plasmid expression vectors containing the alpha-gal cDNA under the control of various regulatory elements. Transfection with a construct containing the myosin light chain 1/3 enhancer in combination with the CMV promoter resulted in higher expression and secretion than with constructs combining the same enhancer with the rabbit beta-myosin heavy chain promoter, or containing only the CMV promoter. Expression of alpha-gal was detectable in mature myotubes in culture at least 10 days after transient transfection, with detectable enzyme activity both in cell extracts and in supernatants. Human fibroblasts deficient in alpha-gal were able to take up the enzyme from medium conditioned by transfected myoblasts via mannose-6-phosphate receptors. The construct that gave the best results *in vitro* was injected in mouse *tibialis anterior* muscles. Injected muscles showed significantly higher levels of alpha-gal than control muscles seven days after injection of DNA, suggesting that this approach could be used for the production of human alpha-gal in vivo.

OVERVIEW SUMMARY

Lysosomal storage disorders are amenable to treatment by enzyme replacement. Genetic modification of muscle via direct injection of expression vectors might represent an alternative method of providing the defective enzymes, if adequate and long-lasting expression levels can be achieved in muscle. This study presents data on the expression and secretion of human alpha-galactosidase by a myogenic cell line *in vitro* and by muscle fibres *in vivo*.

INTRODUCTION

Anderson-Fabry disease is a lysosomal storage disorder (LSD) resulting from the deficiency of the lysosomal enzyme alpha-galactosidase (alpha-gal, EC 3.2.1.22). This enzymatic defect leads to the deposition of neutral glycosphingolipids in most tissues, the pathological and clinical manifestations of the disease being the result of progressive accumulation in endothelial cells leading to ischemia and infarction in organs like kidney, heart or brain. Medical management of this disease includes symptomatic relief of the pain, dialysis and renal transplantation (Desnick et al., 1995).

The discovery of the mannose-6-phosphate receptor pathway (Sly, 1985) has revealed that in addition to the sorting mechanisms operating in the trans-golgi network, lysosomal enzymes can also be recaptured from the extracellular space via these receptors. In keeping with this, it has been shown that the administration of purified lysosomal enzymes to the culture medium can correct the enzymatic defect in fibroblasts from patients with various types of LSD (Neufeld, 1991; Salvetti et al., 1995a). This ability of cells to take up the enzyme has provided the basis for the use of replacement therapy for this group of disorders. In the case of Fabry disease, early studies showed that alpha-gal partially purified from various sources is taken-up by skin fibroblasts from Fabry hemizygotes when added to the culture medium and does catabolize the accumulated substrate, globotriaosylceramide (CTH). This prompted several clinical trials of enzyme replacement in the 1970's which demonstrated the feasibility of enzyme therapy for Fabry disease (Desnick et al., 1995). The unavailability of large amounts of the purified human enzyme has prevented a proper evaluation of the efficacy of replacement therapy so far. Recent efforts using various expression systems to generate large amounts of alpha-gal suitable for enzyme

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replacement clinical trials might make this possible (Coppola et al., 1994). However, it is not known what the half-life of the recombinant protein will be and how frequently the protein will need to be injected in order to remove and prevent glycosphyngolipid deposition.

Alternative ways of providing a source of active enzyme for the treatment of LSD have included bone marrow transplantation and, more recently, gene transfer into haematopoietic stem cells or enzyme delivery into the whole organism by genetically modified cells. For instance, it has been recently shown that fibroblasts transfected with retroviral vectors and grown in collagen lattices which were implanted in the peritoneal cavity successfully secreted beta-glucuronidase and corrected the storage lesions in the liver and spleen of Mucopolysacharidosis VII mice (Moullier et al., 1993b). The same approach resulted in long-term secretion of this enzyme in dogs (Moullier et al., 1995). Similar results were obtained in nude mice transplanted with neo-organs which were secreting alpha-L-iduronidase (Salvetti et al, 1995b).

Since the discovery that skeletal muscle can be transfected *in vivo* by intramuscular injection of plasmid DNA (Wolff et al., 1990, 1991, 1992), this organ system has attracted considerable attention as a potential source of secreted therapeutic proteins. Injection of plasmid DNA constructs has been used successfully for the expression of dystrophin (Acsadi et al., 1991), factor VII (Miller et al., 1995), apolipoprotein-E (Fazio et al, 1994) and decorin (Isaka et al, 1996), whereas the intramuscular injection of genetically modified myoblasts gave encouraging results in the secretion of human growth hormone (Dhawan et al., 1991), factor IX (Dai et al., 1992), beta-glucuronidase (Naffakh et al, 1996), human and murine erythropoietin (Hamamori et al,, 1994; Naffakh et al., 1996), and human glucocerebrosidase (Bansal et al., 1994). However, the

efficiency of these methods of transfection is still low, even with the induction of degeneration and regeneration through injection of myotoxic substances prior to the injection of DNA (Davis et al., 1993a; Wells, 1993; Vitadello et al., 1994). Although direct plasmid injection in muscle is superior to viral vectors (Davis et al, 1993b) and is considered to be sufficient for other purposes like DNA-based immunization, in most cases the levels of expression reported were not high enough to increase the blood levels of circulating proteins. However, the safety, simplicity and low-cost of intramuscular injection of plasmid DNA make it a very attractive alternative to other methods. Together with various modifications aimed at increasing the potency of these vectors and at minimising the immune response against transfected myofibres (Hartikka et al., 1996; Sato et al. 1996), the introduction of muscle-specific regulatory elements could help to improve the levels of expression achieved in muscle *in vivo*.

In the present study, we have created expression vectors containing various combinations of general and muscle-specific promoters with a muscle-specific enhancer. We have analysed the expression and secretion of human alpha-galactosidase from cultured myoblasts after liposome-based transient transfection with these constructs, as well as the production of human alpha-gal in mature mouse muscle after direct injection of the construct showing the highest expression *in vitro*.

METHODS

Expression vectors

The composition of the expression vectors used in this study are summarized in Table 1. The cDNA coding for alpha-galactosidase was amplified by RT-PCR and cloned in pCRII™ (Invitrogen) (Novo et al., 1995), resulting in pGal-wt. Nsi I digestion of this construct releases a fragment comprising the alpha-gal cDNA (including 60 bp of 5'-UTR) flanked by sequences from the multiple cloning site of pCRII™. This fragment which was cloned in the Pst I site of pβPASe9 (Miller et al, 1995) in order to generate the construct pMCagalF. Alternatively, EcoRI digestion of pGal-wt releases the alpha-gal cDNA without flanking sequences, and this fragment was cloned in pcDNA3 (Invitrogen) for the generation of pIVGF, pX3F and pX4F (see Table 1 for details). A different fragment containing the cDNA for alpha-gal with only 25 bp of 5'-UTR and no flanking sequences (gift from Dr. H. Sakuraba) was used in the construction of pX7F. All these constructs contain different combinations of either a truncated rabbit beta-cardiac myosin heavy chain (MHC) promoter (Cribbs et al., 1989) or the CMV promoter with a myosin light chain 1/3 (MLC1/3) enhancer (Donoghue et al, 1988), as shown in Table 1. In constructs pX3F, pX4F and pX7F, the MLC1/3 enhancer was cloned in the Bgl II site of pcDNA3. The reporter plasmid pCMVβ (Clontech) contains the bacterial beta-galactosidase under the control of the CMV promoter (gift from Dr. G. Dickson).

Transfection of myogenic cells *in vitro*

DNA for transfections was prepared using Plasmid midi-columns (Qiagen). C2C12 mouse myoblasts (purchased from the European Collection of Animal Cell Culture) were plated at 1.5

x 10^4 cells/cm^2 and grown overnight in growth medium (DMEM/10%FCS with penicillin-streptomycin-amphotericin B). All plates were coated with rat tail Type I collagen (Sigma). For 35-mm plates, transfections were performed mixing 10 μg of Lipofectamine (Gibco) with 2 μg of DNA in 200 μl of Optimem-1 (Gibco) following conditions recommended previously (Trivedi et al., 1995). After 30 min incubation at room temperature, the mixture was diluted up to 1 ml in Optimem-1 and added to the cells. Transfections were carried out for 6-8 hours at 37°C/5%CO$_2$ and included pCMV-β (typically 200 ng in 2μg of total DNA) as an internal control of transfection efficiency. After transfection, plates were washed with PBS followed by addition of DMEM/2% horse serum (differentiation medium). Under these conditions, myoblasts start the process of fusion and differentiation into myotubes, which become visible after 48 hours and continue to develop for 4-6 days.

For the preparation of cell extracts, cells were washed twice with PBS, scraped in 500 μl of cold PBS, pelleted and resuspended in 100 μl of Reporter Lysis Buffer (Promega). After vortexing for 15 min. at room temperature and spinning for 3 min at 4°C, supernatants were stored at -70°C. Alpha-gal and beta-gal were quantitated in a TKO 100 fluorometer (Hoeffer Scientific Instruments) using fluorometric assays with the corresponding 4-methyl-umbelliferyl substrates. Alpha-gal was assayed using N-acetyl-galactosamine (0.1M final concentration in the reaction) to avoid the interference of N-acetyl-galactosaminidase (Mayes et al., 1981), and the enzymatic activity was expressed in Units/L (1 Unit=1 nmole of 4-methyl-umbelliferone per hour). Beta-gal activity (1 Unit=1μmole of 4-methyl-umbelliferone per hour) was assayed following the protocol provided by the manufacturer of the fluorometer. Total proteins were measured by the bicinchonic acid method (Sigma) using bovine serum albumin as standard. For all transfection

experiments, unless otherwise stated, alpha-gal activity of transfectants (in Units/mg of protein) was calculated after subtracting the activity of the controls (no-DNA transfectants) and the same was done for beta-gal. Results were expressed as Units of alpha-gal/Unit of beta-gal. Alpha-gal enzymatic activity in supernatants was expressed in Units/L.

Culture of Fabry fibroblasts in conditioned medium

Human Fibroblasts from a hemizygous Fabry disease patient (gift from Dr. B. Winchester) were plated at $5x10^3$ cells/cm² and cultured in DMEM/15% FCS for 2-4 days. The medium was then replaced by DMEM/2% horse serum that had been conditioned for 24 hours by myoblasts transfected with pX7F as described above. The conditioned medium was renewed after 48 hours. Conditioned media were 0.22μm-filtered before being added to the fibroblasts in order to avoid carry-over of the liposome-DNA complex. Fibroblasts were harvested after a total of 3 days incubation in conditioned medium, and cell extracts prepared and assayed as described for C2C12 myoblasts.

Injections and preparation of muscle extracts

Animal studies were performed on 5-6 week-old C57Bl/6 male mice (Wells and Goldspink, 1992), kept under standard conditions. Mice were anaesthesised with Hypnorm/Diazepam and injected in the *tibialis anterior* muscles with 30 μg of DNA in 50 μl of sterile, endotoxin-free saline, following previous recommendations (Manthorpe et al., 1993). DNA for intramuscular injections was prepared using the Endo-free Plasmid Kit (Qiagen), and resuspended in endotoxin-free sterile saline. DNA concentration and purity were assessed by UV spectroscopy. Some muscles were pre-injected with 1.2% $BaCl_2$ (Dr. D. Wells, personal communication) or with

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0.1M cardiotoxin from *Naja nigricollis* (Latoxan) 5 days prior to the injection of DNA, in order

to induce myotoxicity and a cycle of degeneration/regeneration (Davis et al., 1993a). Seven days

after injection of DNA the animals were sacrificed and the muscles were dissected and frozen at

-70°C, finely ground on a pre-cooled mortar and then vortexed for 15 min at room temperature

in 500 μl of Reporter Lysis Buffer (Promega), spun for 3 min. at 4°C and the supernatants stored

at -70°C. Proteins and alpha-gal enzymatic activity were determined as described above, and

results were expressed in Units/mg of protein. Statistical significance of the difference between

two medians was performed using a non-parametric significance test (Mann-Whitney ranks-sum

test for unpaired samples).

RESULTS:

Effect of muscle-specific regulatory elements

In order to select a construct suitable for the study of the expression of alpha-gal after intramuscular injection, we first evaluated the efficiency of expression and secretion of human alpha-gal by the various constructs using an *in vitro* system in which C2C12 mouse myoblasts are transfected and alpha-gal enzymatic activity is measured in cell extracts either after 18-20 hours (undifferentiated myoblasts) or after 5-10 days (big myotubes) in differentiation medium. In a first set of experiments, the construct in which alpha-gal expression is driven by the CMV promoter (pIVGF in Fig. 1 A) showed the highest activity in undifferentiated myoblasts (18 hours post-transfection), followed by pX4F and pMCagalF in this order. In contrast, the analysis of fully differentiated 10-day myotubes (Fig. 1, B) revealed that alpha-gal enzymatic activity was clearly higher in pX4F than in pIVGF, the only difference between both constructs being the presence of the MLC1/3 enhancer in pX4F. Furthermore, pMCagalF, which contains both the MHC promoter and the MLC1/3 enhancer, gives similar levels of expression to pIVGF in differentiated myotubes.

We also compared the expression levels generated by pX3F, pX4F and pX7F, which only differ in the orientation of the MLC1/3 enhancer (pX3F and pX4F) or in the length of the 5'-UTR of the alpha-gal cDNA (shorter in pX7F than in pX3F and pX4F), both constructs having the MLC1/3 enhancer in the same orientation). pX3F and pX4F showed the same activity after 6 days in differentiation medium (results not shown), and all three constructs gave very similar results after 48 hours in differentiation medium, as shown in Fig. 2. Construct pX7F was chosen for further experiments.

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The inclusion of pCMV-β in all transfections allowed to correct for plate-to-plate differences in transfection efficiency. This is clearly shown in Fig. 1, where the discrepancy between duplicates is much higher when only alpha-gal activity is considered (expressed in Units/mg of protein in cell extracts) than when these values are divided by the corresponding beta-gal activity (also in Units/mg) of each cell extract. The results for pX4F in Fig. 1B are particularly illustrative of this effect, and emphasize the importance of using an internal control for transfection efficiency when comparing different constructs, since slight variations in the quality of the DNA or in the growth conditions of the cells are important sources of experimental variability.

Secretion of human alpha-gal to the culture medium and uptake by alpha-gal-deficient fibroblasts

We also evaluated the secretion of alpha-gal to the culture medium after transient transfection of C2C12 myoblasts. Fig. 3 shows the total activity of alpha-gal in cell extracts and in supernatants conditioned for 48 hours after transfection with pX3F, pX4F and pX7F, compared to the controls (mock transfected, no DNA). The total amount of alpha-gal activity in the culture medium of transfected cells was higher than in controls (average of the three constructs=2.17 Units vs. 0.10 Units in controls). Furthermore, the percentage of alpha-gal in the medium relative to the cell extract was also increased in transfected cells compared to controls (average of 18.7% vs. 6.5%, respectively), indicating that the enzyme produced after transfection is being efficiently secreted. Again, all three constructs tested gave comparable results.

In order to confirm that the alpha-gal expressed and secreted *in vitro* has undergone correct post-translational processing, we investigated the ability of alpha-gal-deficient fibroblasts to take up

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the enzyme from medium conditioned by C2C12 myoblasts transfected with pX7F. Fibroblasts from a hemizygous Fabry patient were cultured for 4 days in differentiation medium that had been conditioned by C2C12 myoblasts transfected with pX7F. The conditioned medium was filtered through 0.2μm syringe filters before being added to the fibroblasts, in order to avoid any carry-over of remaining liposome-DNA complexes. Fig. 4 shows the alpha-gal enzymatic activity in fibroblasts under these conditions, with significantly higher levels in those cultured with medium conditioned by pX7F-transfected myoblasts than in those cultured with medium conditioned by mock-transfected myoblasts ($p < 0.01$). However, this effect was completely abolished by the addition of mannose-6-phosphate (5mM) to the conditioned medium, as shown in Fig. 4. This strongly suggests that this increase in alpha-gal activity was the result of uptake of the enzyme via mannose-6-phosphate receptors.

Production of human alpha-gal after intramuscular injection of plasmid DNA

The activity of alpha-gal was also analysed in muscle extracts from mice injected intramuscularly with DNA. When we pre-injected the *tibialis anterior* muscles with $BaCl_2$ or with cardiotoxin from *Naja nigricollis*, which have been shown to increase the transfection efficiency of intramuscular injection of DNA, we observed an increase in alpha-gal in both injected and control muscles, with no significant differences between them. In contrast, injection of pX7F DNA in muscles that had not been pre-injected with myotoxic substances resulted in significantly increased levels of alpha-gal activity with respect to the control muscles injected with saline ($p < 0.01$) 7 days after injection (Fig. 5).

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DISCUSSION:

Development of vectors for *in vitro* expression of human alpha-galactosidase

Most clinical manifestations of Fabry disease arise from globotriaosylceramide deposition in endothelial and smooth muscle cells of small vessels. Early enzyme replacement clinical trials showed that systemic delivery of the enzyme can decrease the sphyngolipid levels in plasma and prevent cellular storage (reviewed in Desnick et al., 1995). Sustained secretion of the enzyme from genetically modified muscles could represent a more efficient way of achieving this goal. In order to obtain an expression vector suitable for production of human alpha-gal in muscle after intramuscular injection of plasmid DNA, we first used an *in vitro* system to compare various constructs in which the expression of alpha-gal is modulated by different regulatory elements. The fact that C2C12 mouse myoblasts undergo fusion and differentiation under certain culture conditions, thereby switching on the expression of muscle-specific genes, provides a good system to study the relative efficiency of the vectors used for intramuscular injection. Using this system we have compared two constructs which are identical except for the presence or absence of the MLC1/3 enhancer. Our results clearly show that the MLC1/3 enhancer increases the strength of the expression driven by the CMV promoter in an orientation-independent manner in differentiated myogenic cells, but has no effect in undifferentiated myoblasts (Fig. 1A). This confirms previous data showing that this enhancer confers muscle-specificity to a SV40 promoter and that its activity is orientation-independent (Donoghue et al., 1988), and suggests that muscle differentiation provides the necessary muscle-specific factors for this element to exert its function of enhancing the basal activity of heterologous promoters. It is particularly interesting to note that the construct lacking the MLC1/3 enhancer showed a marked decline in the total alpha-gal activity (in Units/mg) after 10 days following transfection, as would be expected from transient

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transfection experiments (pIVGF in Fig. 1 A and B). In contrast, both constructs containing this enhancer maintain constant levels of alpha-gal production for at least 10 days following transient transfection (middle bar for pMCagalF and pX4F in Fig. 1 A and B). Since the levels of beta-gal decline with time after transfection (beta-gal expression is driven by a CMV promoter in the plasmid pCMVβ), the net result is an increase in the normalized alpha-gal activity (in Units alpha-gal per unit of beta-gal) in fully differentiated myotubes as compared to undifferentiated myoblasts, but this reflects the decrease in beta-gal rather than a true increase in alpha-gal expression. The fact that myoblasts can maintain constant levels of alpha-gal expression for at least several days in culture after transient transfection with vectors containing the MLC1/3 enhancer offers the possibility that this element will increase and prolong expression levels in mature muscle fibres *in vivo*.

Our results suggest that the rabbit beta-MHC promoter, although very specific for expression in mature muscle, is weaker than the CMV promoter in undifferentiated myogenic cells *in vitro*. When the CMV promoter is combined with the MLC1/3 enhancer in the same expression vector this gave stronger expression in myotubes than using the MHC promoter. As shown in Fig. 1B, pX4F results in levels of alpha-gal expression 3 times higher than pMCagalF, both constructs differing only in the promoter (CMV promoter in pX4F or beta-MHC promoter in pMCagalF). These results indicate that muscle-specific regulatory elements combined with strong viral promoters in expression vectors can potentiate the activity of the promoter under conditions that simulate mature muscle fibres.

Secretion and uptake of alpha-galactosidase *in vitro*

It has been previously shown that overexpression of alpha-gal in CHO cells results in increased secretion of the enzyme to the culture medium (Desnick et al., 1995). The expression levels we have achieved in C2C12 myoblasts have also been sufficient to increase the concentration of the enzyme in the culture medium, and in fact the relative increase in alpha-gal activity in supernatants exceeded the one in cell extracts. This is reflected in the percentage of secreted alpha-gal (total enzyme activity in supernatants relative to cell extracts) which is higher in transfected myoblasts (20.3%, 16.1% and 19.6% for pX3F, pX4F and pX7F, respectively) than in mock-transfected cells (6.5%, see Fig. 3).

In order to ascertain whether the secreted alpha-gal can be taken up by human fibroblasts, we cultured fibroblasts from a Fabry patient in medium conditioned by transiently transfected myoblasts that were secreting human alpha-gal. After three days in these conditions, the alpha-gal-deficient fibroblasts showed a significant increase in the enzymatic activity in the cell extracts (Fig. 4), suggesting an uptake mechanism. Inhibition of this uptake by the addition of mannose-6-phosphate to the medium strongly argues in favour of the mannose-6-phosphate receptor pathway as the responsible mechanism, as has been shown before for this and other lysosomal enzymes. The fibroblasts from a hemizygous Fabry patient used here had a very low alpha-gal enzymatic activity, so that in this low background it was easier to detect the increase due to the uptake of the enzyme from the culture medium. Using immunostaining with a monoclonal antibody anti-CTH (anti-globotriaosylceramide) we have tried to assess whether the increase in alpha-gal detected in these fibroblasts could be sufficient to remove the storage deposits, but we could not observe any dramatic changes in staining intensity (results not shown). It is likely that a longer period of culturing in conditioned medium is required for the disappearance of the stored

globotriaosylceramide to be detected by this method, but maintaining alpha-gal-deficient fibroblasts for a long time in these culture conditions poses technical problems that have not yet been circumvented.

Prospects for *in vivo* production of human alpha-gal from muscle

Injection of a plasmid DNA construct combining a strong viral promoter with a muscle-specific enhancer in mouse *tibialis anterior* muscles resulted in significantly higher levels of the enzyme as compared to control muscles injected with saline one week after injection (Fig. 5). Expression of foreign proteins in muscle has been shown to elicit an immune response against those fibres expressing the protein (Vitadello et al., 1994), resulting in the number of transfected fibres decreasing dramatically with time. Furthermore, other studies have shown that luciferase levels decrease sharply 14 days after injection of plasmid constructs (Hartikka et al., 1996). Therefore we only allowed expression to proceed for one week after injection, in order to avoid a potential immune response against human alpha-gal. We are currently in the process of constructing expression vectors in which the human alpha-gal cDNA is replaced by the mouse cDNA, in order to test the stability of expression in transfected mouse muscles for longer periods of time.

Although it has been previously shown that pre-injection with myotoxic substances increases the efficiency of direct muscle injection, we have found that this procedure leads to abnormally high levels of alpha-gal, probably due to the degenerative process associated with it. In this higher background it is more difficult to detect any increase in enzyme activity resulting from the gene transfer procedure, especially in animals with normal levels of the enzyme. It would be interesting to see whether other lysosomal enzymes show a similar behaviour in muscle forced

into a cycle of degeneration/regeneration.

The fact that human alpha-gal secreted by differentiated myoblasts was readily taken-up by human fibroblasts from a Fabry patient suggests that the enzyme is correctly glycosylated. Furthermore, there was an active uptake from the culture medium even though the enzyme levels achieved in supernatants were not high. Although we could not detect increased enzymatic activity in the plasma of animals expressing human alpha-gal in their muscles (results not shown), this could be due to short plasma half-life (the half-life of infused purified enzyme was between 10 min. and 70 min. in two previous enzyme replacement clinical trials).

Studies in which lysosomal enzymes were secreted from genetically engineered neo-organs have shown increased enzymatic activity in several organs, although increased plasma levels were not reported (Moullier et al., 1993a; 1993b; 1995). This might suggest an active uptake mechanism from the circulation even with normal plasma levels. Likewise, expression of human apo-E2 in rat muscle only resulted in increased plasma levels in an animal model in which the clearance of the protein from the circulation was impeded due to low numbers of LDL receptors (Fazio et al., 1994). We could not observe a significant increase in alpha-gal activity in the liver of animals expressing the enzyme in muscle (results not shown), but the short time of the experiments (one week) and the endogenous high levels of the enzyme in the liver of normal mice might account for this.

Our results suggest that human alpha-gal can be expressed and secreted from muscle cells and taken-up by human cells which are deficient in this enzyme. Direct intramuscular injection of one

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expression vector generated in this study resulted in high enzyme levels in injected muscles, but the assessment of the curative potential of this approach will require long-term animal studies using the mouse enzyme in order to avoid immunological rejection of the transfected muscle fibres expressing human alpha-gal.

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CONSTRUCT	PARENT VECTOR	PROMOTER	ENHANCER
pMCagalF	pβPASe9	MHC	MLC1/3
pIVGF	pcDNA3	CMV	---
pX3F and pX7F	pcDNA3	CMV	MLC1/3 (sense)
pX4F	pcDNA3	CMV	MLC1/3 (antisense)

Table 1: Details of the expression vectors used in this study. MHC=β-cardiac myosin heavy chain. MLC1/3=myosin light chain 1/3. Constructs pX3F, pX4F and pX7F contain the MLC1/3 enhancer cloned either in the direction of transcription (sense) or in the reverse orientation (antisense) as indicated. Constructs pX3F and pX7F differ in the length of the 5'-UTR included in the cDNA (see text for details).

FIGURE LEGENDS

Figure 1: Comparison of constructs pIVGF, pX4F and pMCagalF after transfection of C2C12 myoblasts (see Table 1 for details of constructs). Enzymatic activity of beta-gal and alpha-gal (in Units/mg of protein, left axis) and the normalized alpha-gal activity (in Units alpha-gal/Unit beta-gal, right axis) are shown either 18 hours post-transfection (undifferentiated myoblasts, A) or 10 days post-transfection (fully differentiated myotubes, B). High-Low bars show the results from duplicate experiments.

Figure 2: Comparison of constructs pX3F, pX4F and pX7F after transfection of C2C12 myoblasts (see Table 1 for details of constructs). Enzymatic activity of beta-gal and alpha-gal (in Units/mg of protein, left axis) and the normalized alpha-gal activity (in Units alpha-gal/Unit beta-gal, right axis) 48 hours post-transfection (small myotubes) are shown. High-Low bars show the results from duplicate experiments.

Figure 3: Total alpha-gal enzymatic activity (in Units, 1 Unit = 1 nmole/h) of cell extracts and of supernatants from C2C12 myoblasts transfected with three different constructs (Mock = no-DNA transfected) and harvested 48 hours after transfection. Total alpha-gal activity was derived from the original enzymatic activity in cell extracts (in Units/mg) or in supernatants (in Units/L). High-Low bars show the results from duplicate experiments.

Figure 4: Alpha-gal activity in cell extracts from fibroblasts of a Fabry patient that were cultured for 3 days in medium conditioned by C2C12 myoblasts transfected as indicated, either in the absence or in the presence (+M6P) of 5mM mannose-6-phosphate in the culture medium. Error bars = S.E.M. (n=6).
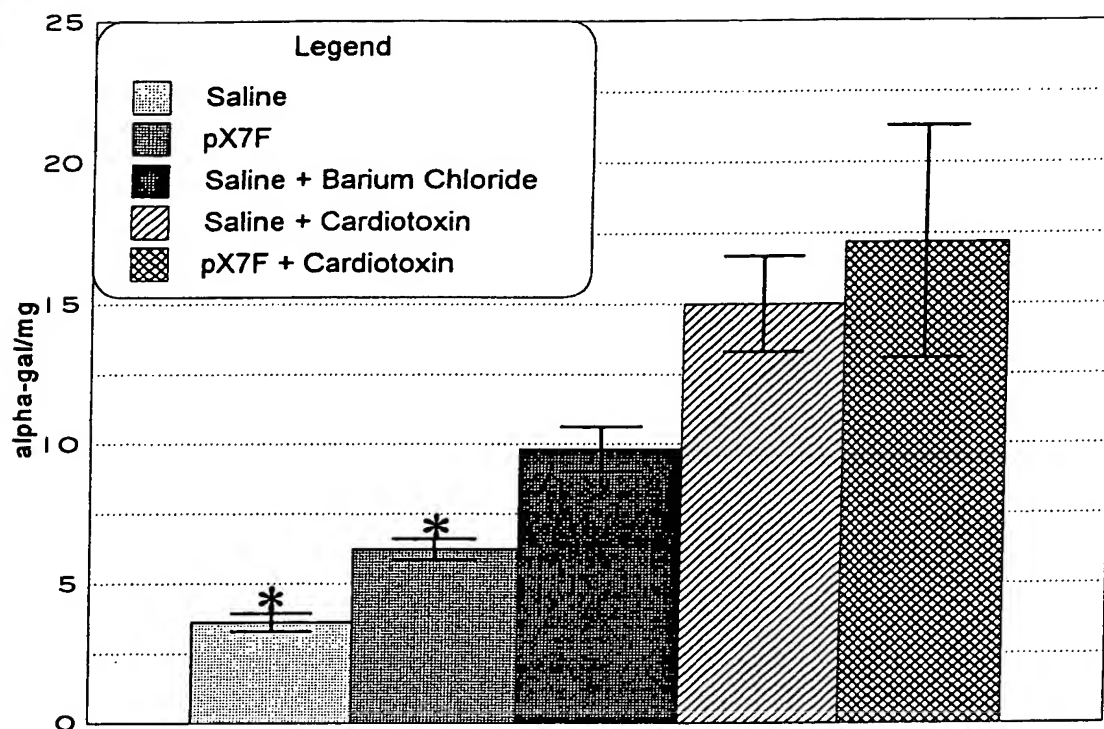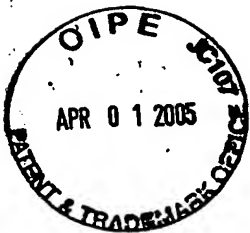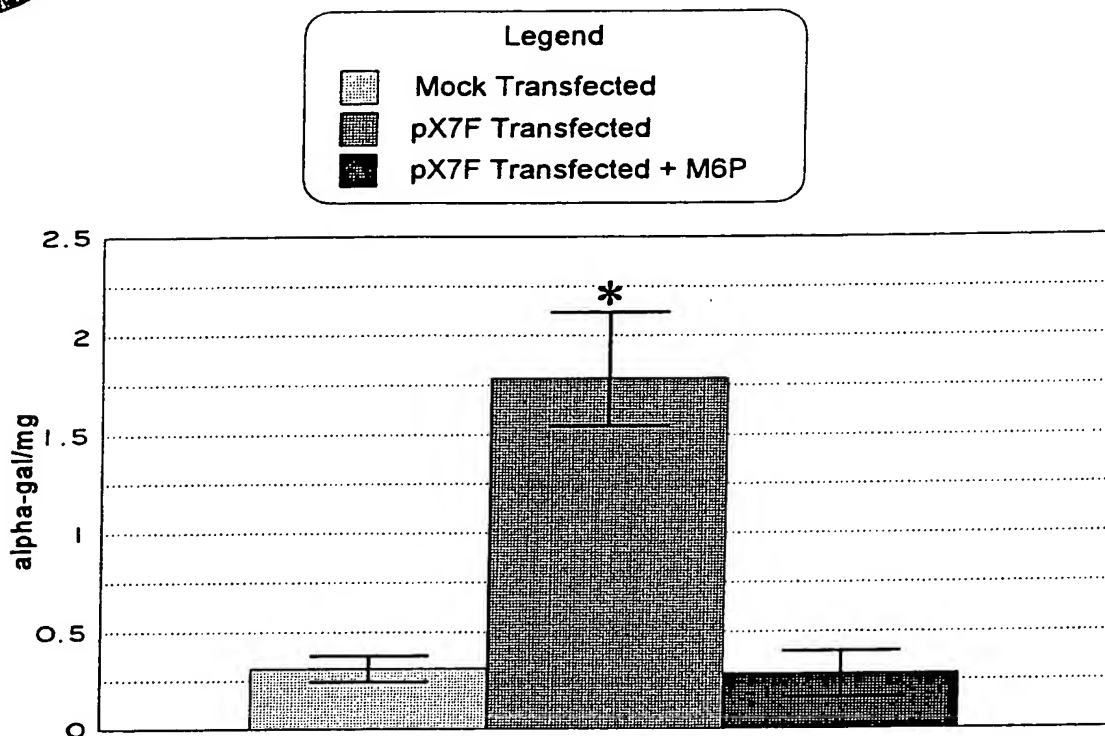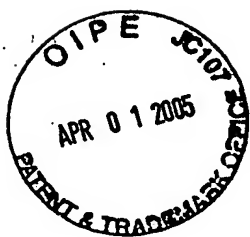*Significant difference (p < 0.01) with any of the other groups.

Figure 5: Alpha-gal activity in tibialis anterior muscle extracts 7 days after injection. 30 μg of pX7F DNA in 50 μl of sterile, endotoxin-free saline (or 50 μl of saline in control muscles) were injected in tibialis anterior muscles of 5-6 week-old C57Bl/6 mice. Some muscles were pre-injected with myotoxic substances (1.2% $BaCl_2$ or 0.1M Cardiotoxin from Naja nigricollis) 5 days prior to the injection of DNA, in order to induce a cycle of degeneration/regeneration. Error bars = S.E.M. (n=6).

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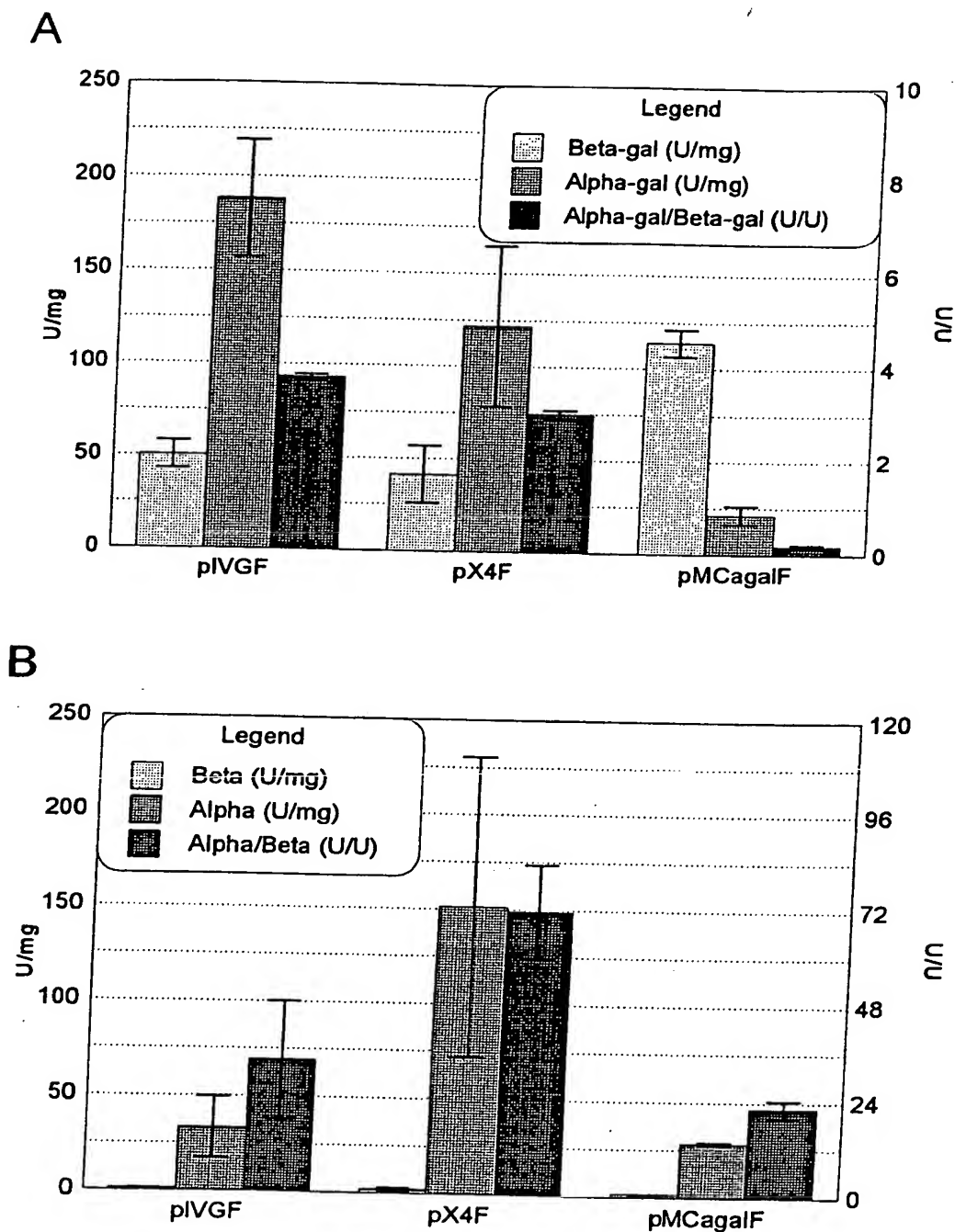*Significant difference ($p < 0.01$) with any of the other groups.
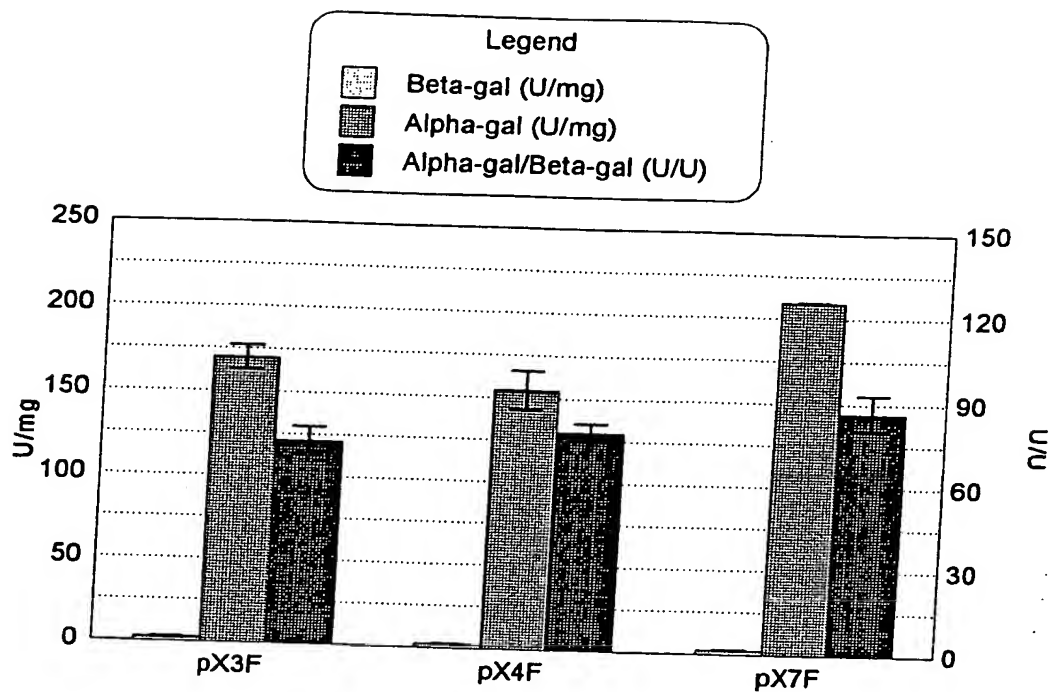
A

B

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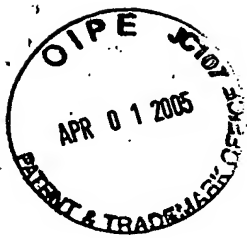
Figure 2: Comparison of constructs pX3F, pX4F and pX7F after transfection of C2C12 myoblasts (see Table 1 for details of constructs). Enzymatic activity of beta-gal and alpha-gal (in Units/mg of protein, left axis) and the normalized alpha-gal activity (in Units alpha-gal/Unit beta-gal, right axis) 48 hours post-transfection (small myotubes) are shown. High-Low bars show the results from duplicate experiments.
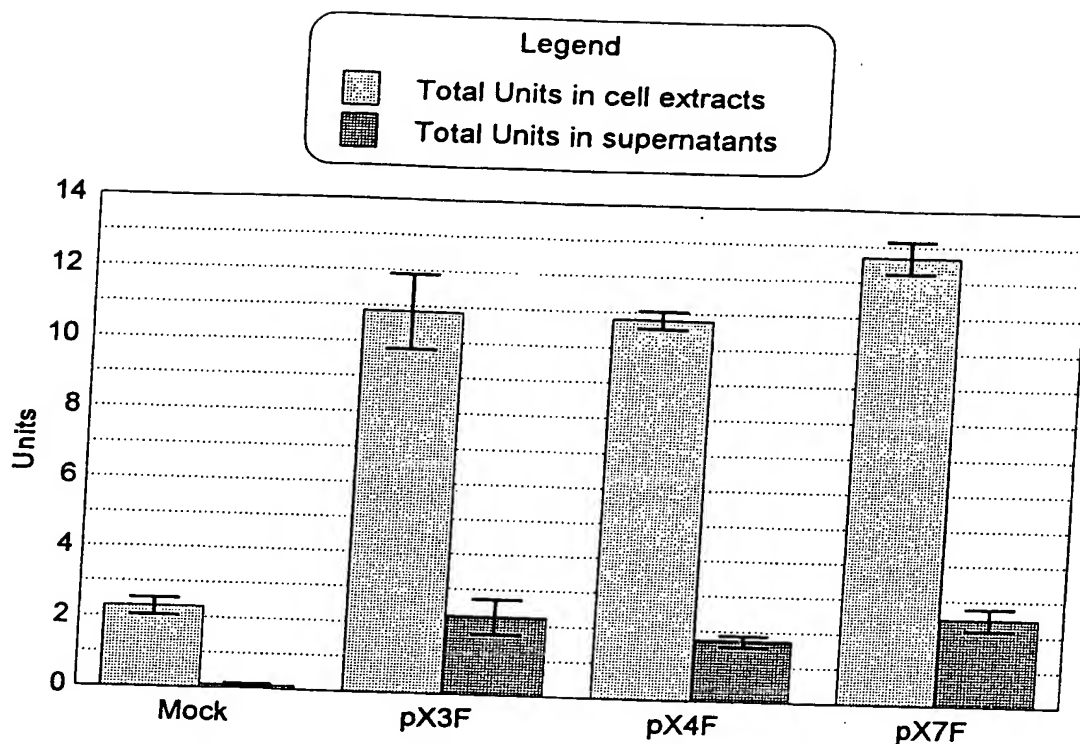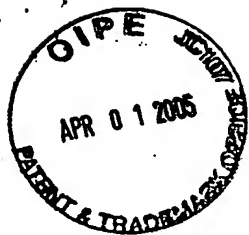
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